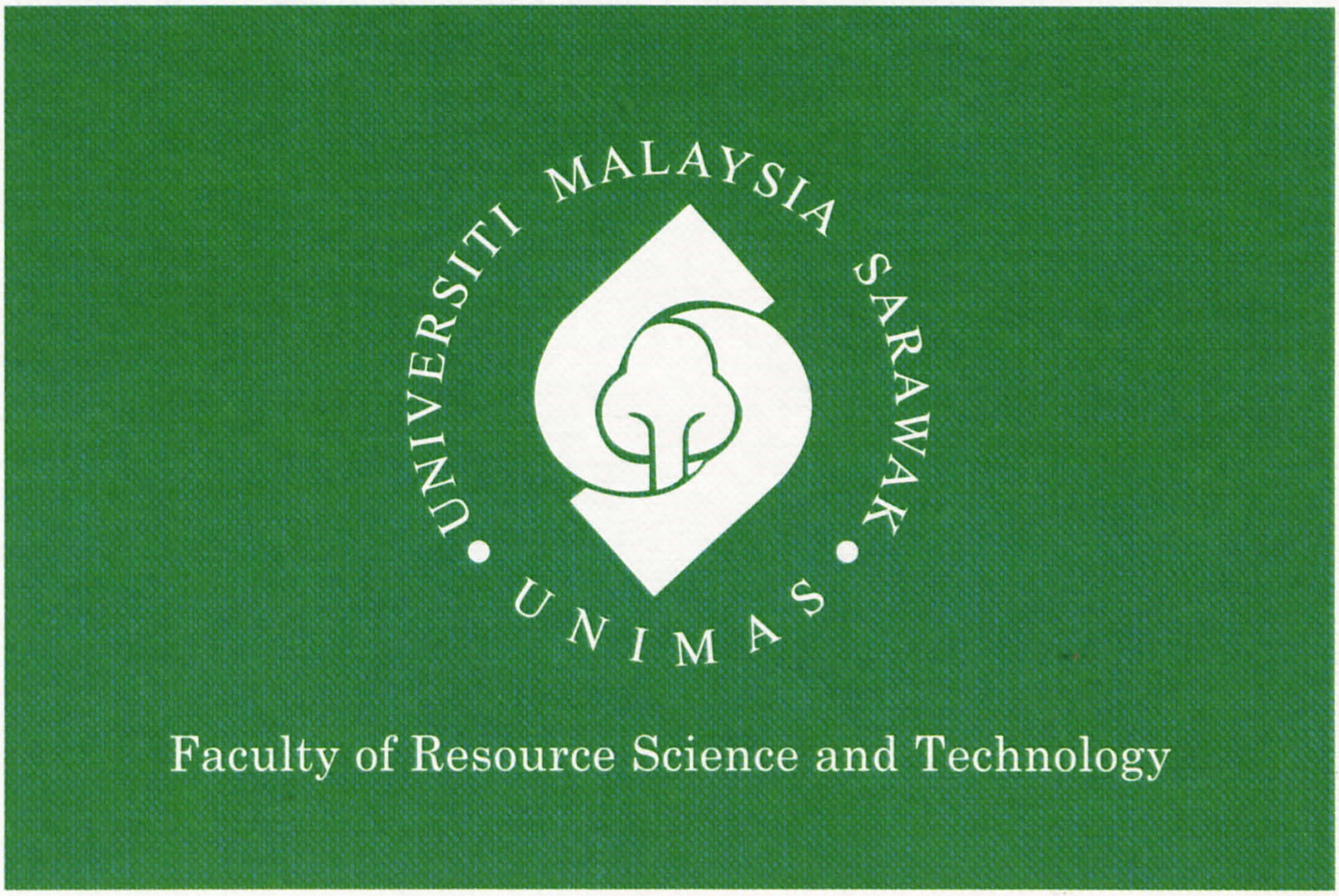

Isolation and Cloning of *ABCH1* Gene from *Rasbora sarawakensis*

Arin Vynona Robert
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Bachelor of Science with Honours
(Resource Biotechnology)
2016

Cloning and Expression of *ABCH1* Gene from *Rasbora sarawakensis*

ARIN VYNONA ROBERT (40614)

A thesis report submitted in partial fulfillment of the Final Year Project 2 (STF 3015) Resource Biotechnology

Supervisor: Dr. Chung Hung Hui

Resource Biotechnology
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17/5/2016

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UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☑

Masters ☐

PhD ☐

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This declaration is made on the .....1.....day of...August.....2016.

Student's Declaration:

I **Arin Vynona Robert** with **matric number of 40614** under **Faculty of Resource Science and Technology** hereby declare that the work entitled **Isolation and Cloning of *ABCH1* gene from *Rasbora sarawakensis*** is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

1/8/2016
Date submitted

ARIN VYNONA ROBERT (40614)
Name of the student (Matric No.)

Supervisor's Declaration:

I **Dr. Chung Hung Hui** hereby certifies that the work entitled **Isolation and Cloning of *ABCH1* gene from *Rasbora sarawakensis*** was prepared by the above named student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of **Bachelor of Science with Honours** and the aforementioned work, to the best of my knowledge, is the said student's work.

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Table of contents

List of Abbreviations

ABCBH1	ATP-binding cassette sub-family H member 1
ATP	Adenosine Triphosphate
bp	Base pair
cDNA	Complimentary Deoxyribonucleic acid
ddH_2O	Deionized Water
dNTP	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic acid
EtBr	Ethidium Bromide
MDR	Multidrug Resistance
NBDs	Nucleotide Binding Domains
NCBI	National Center for Biotechnology Information
%	Percentage
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
TAE Buffer	Tris-Acetic-EDTA
TMDs	Transmembrane Domains
µL	Micro Liter
mL	Mili Liter

List of Figures

List of Tables

Isolation and Cloning of *ABCH1* Gene from *Rasbora sarawakensis*

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Abstract
ABC transporter proteins are vital in living organism as they have significant function in transporting molecules across the biological membrane. They are encoded by the ABC genes which comprised several different classes and members in their superfamily. In fish, *ABCH1* gene might involve in transporting cholesterol or as a multidrug resistance protein. This study aim to identify *ABCH1* gene in *Rasbora sarawakensis* and as well as clone it into pGEM-T vector. Extraction of total RNA was done from the whole fish sample using TRI reagent and phenol chloroform precipitation. The cDNA was amplified using designed primer after generated by reverse transcription with PCR. The amplicon with size of 768 bp was then cloned into pGEM-T Easy Vector (Promega). Subsequently, transformation was made using in house prepared E. coli XL-1 Blue competent cells which produced 4.4334 X10^4 transformants per ug. Double confirmation of the presence of the insert gene was done by colony PCR after the blue white screening. Restriction digestion by using *NotI* was performed afterward for further confirmation of the insert gene which showed two discreet bands. Sequencing was done after that and the result has proved that *ABCH1* gene is present in *R. sarawakensis* as nBLAST has showed 89% similar with *ABCH1 Danio rerio*. This study gives significant values for future study which will provide better understanding on *ABCH* gene.

Keywords: *R. sarawakensis*, ABC transporter, *ABCH1*, PCR, cloning

Abstrak
ABC pengangkut protein penting dalam organisma hidup kerana mereka mempunyai fungsi penting dalam mengangkut molekul seluruh membran biologi. Mereka dikodkan oleh gen ABC yang terdiri daripada beberapa kelas dan ahli-ahli yang berbeza dalam superfamili mereka. Dalam ikan, ABCH1 gen mungkin terlibat dalam mengangkut kolesterol atau sebagai protein rintangan pelbagai dadah. Tujuan kajian ini adalah untuk mengenal pasti gen ABCH1 dalam Rasbora sarawakensis dan juga mengklon gen itu ke vektor pGEM-T. Pengekstrakan daripada jumlah RNA telah dilakukan daripada sampel ikan seluruh menggunakan reagen-TRI dan kloroform fenol. cDNA telah diamplifikasikan selepas transkripsi terbalik daripada RNA menggunakan primer yang telah dihasilkan dengan PCR. Amplikon dengan saiz 768 bp kemudian diklon ke dalam pGEM-T mudah Vector (Promega). Selepas itu, transformasi dibuat menggunakan E. coli XL-1 Blue sel kompeten. Pengesahan kedua kehadiran gen dilakukan dengan koloni PCR selepas transformasi. Hasil pencernaan restriksi yang dilakukan seterusnya dengan menggunakan NotI untuk pengesahan lanjut gen sisip telah menunjukkan dua band. Plasmid kemudiannya dihantar untuk proses jujukan dan hasilnya telah membuktikan bahawa ABCH1 gen hadir dalam R. sarawakensis setelah diproses dalam nBLAST bahwa terdapat persamaan tertinggi dengan ABCH1 Danio rerio. Kajian ini memberi asas untuk masa depan analisis tentang level ekspresi dan fungsi gen ini dan seterusnya membolehkan R. sarawakensis digunakan sebagai model ekotoksikologi untuk mengkaji keadaan air di Sarawak.

Kata kunci: R. sarawakensis, pengangkut ABC, ABCH1, PCR, kloning

1.0 INTRODUCTION

1.1 Background

Cytoplasmic membrane is highly impermeable to most essential molecule and they separate the cellular environment from external surrounding of the cell. It only allows certain essential ions, proteins or other molecules to be kept in the cells or remain outside of the cells. Thus, in order to maintain the cellular environment from its surroundings, transporter proteins have been developed to mediate the movement of various components to enter and exit the cells. Controlling the movement of molecules that cross the membrane is important to be maintained as to ensure continuous physiological function of the cells in organism (Schneider & Hunke, 1998).

ABC (ATP binding cassette) gene encodes for active transporter proteins which embedded in the membranes and can be found from the lowliest microbe to human. They play various physiological roles. ABC transporter proteins are one of the largest protein superfamily which classified according to their nucleotide identity. There are eight sub-families of the ABC transporter which categorized from sub-family A to H whereas each sub-family constitute of several members. Sub-family H (ABCH) has only one member which is *ABCH1*. Formerly, ABCH transporter can only be found in insects; such as Drosophila and Anapheles, and in slime mold; *Dictyostelium discoideum*.

The only vertebrate that is believed to have the member of *ABCH* gene is fish. ABCH is a half-transporter which made up of only one NBD and one TMD similar to ABCD and ABCG members. To date, *ABCH* member has been reported on genome vertebrates such as green spotted pufferfish: *Tetraodon nigroviridis*, and zebrafish (Popovic et al., 2010). However, the function of the ABCH member especially in teleost level has not yet well known. In this study, I have chosen *Rasbora sarawakensis* as my model organism because

it is endemic to Sarawak freshwater. *R. sarawakensis* can be found in Batang Kayan and Sungai Sarawak in Sarawak and the Mempawah and Melawi in West Kalimantan. (Liao et al., 2010)

Therefore, the study of *ABCH1* gene in *Rasbora sarawakensis* would give an early basis to study the function and potential of ecotoxicology relevance of ABCH transporter.

The aims of this study are:

1. To isolate *ABCH1* gene from *R. sarawakensis*.
2. To clone the partial fragment of *ABCH1* gene from *R. sarawakensis*.

2.0 LITERATURE REVIEW

2.1 ATP-binding Cassette Proteins

The ATP-binding cassette (ABC) proteins play a vital role in transporting diverse substrates across biological membranes (Hollenstein, 2007). The first discovery of these proteins were from its roles as the multidrug resistance (MDR) protein in the chemotherapeutic treatment, which play role as the barrier for the treatment of malignant tumors in human (Vasiliou et. al, 2009). Transporter proteins comprise two highly conserved function domains including the nucleotide binding domain (NBD), and the transmembrane domain (TMD). The NBD is located in the cytoplasm which can utilize ATP to provide energy to transport variety of molecules through cellular membranes, whereas the TMD is embedded in the lipid bilayer and involved in the translocation of specific substrates. Full transporters consist of four domains, two NBDs and two TMDs, while half transporters have one NBD and one TMD. To form a functional transporter, half-transporters must dimerize as either homo- or heterodimers (Lunckenbach et al., 2014).

The transport cycle is initiated by the interaction of substrate with the TMDs from the intracellular face of the membrane. Substrate binding induces a conformational change in the TMDs which is transmitted to the NBDs to initiate ATP hydrolysis. It is clear that both NBDs are required, and both must hydrolyse ATP (Higgins, 2001).

These proteins were found to be highly conserved in vertebrates' species, which related to the cellular detoxification. Though that normally ABC proteins mediate the movements of various substrates in and out of cell, some ABC transporters have their own roles such as drug transporters and as part of toxicants defense. Some members of the ABC

transporters are non-transporters protein but functions as ion channels, receptors, or involved as transcription and translation factors. (Lunckenbach et al., 2014)

Initially, *ABCB1* gene was found to play a significant role in multidrug resistance mechanism and subsequently being known as permeability glycoprotein (Ppg) (Vasiliou et al., 2009). Ppg divided into two different isoforms of the gene which is multidrug resistance 1 (MDR1), have implicated in drug resistance and multidrug resistance 2 (MDR2), the function has not been known (Loncar et al., 2010). Multidrug resistance associated protein 1 (*MRP*1) is another ABC family member which was found to have a wide range in anticancer drug transport activity and they are highly expressed in many human tissues and cancer cells. In contrast with the MDR1, MRP1 is a transporter of the negatively charged natural-product drugs and drugs that have been modified by conjugation, glucuronylation, glutathione and glucosylation sulfation (Gottesman, 2002).

High level of drugs accumulation in the cell was discovered when ABCB1 gene has been knock-out compared to the cells which transfected with *ABCB1* gene. This study proved that the *ABCB1* gene responsible in transporting the drugs out of the cells (Jeong et al., 2014).

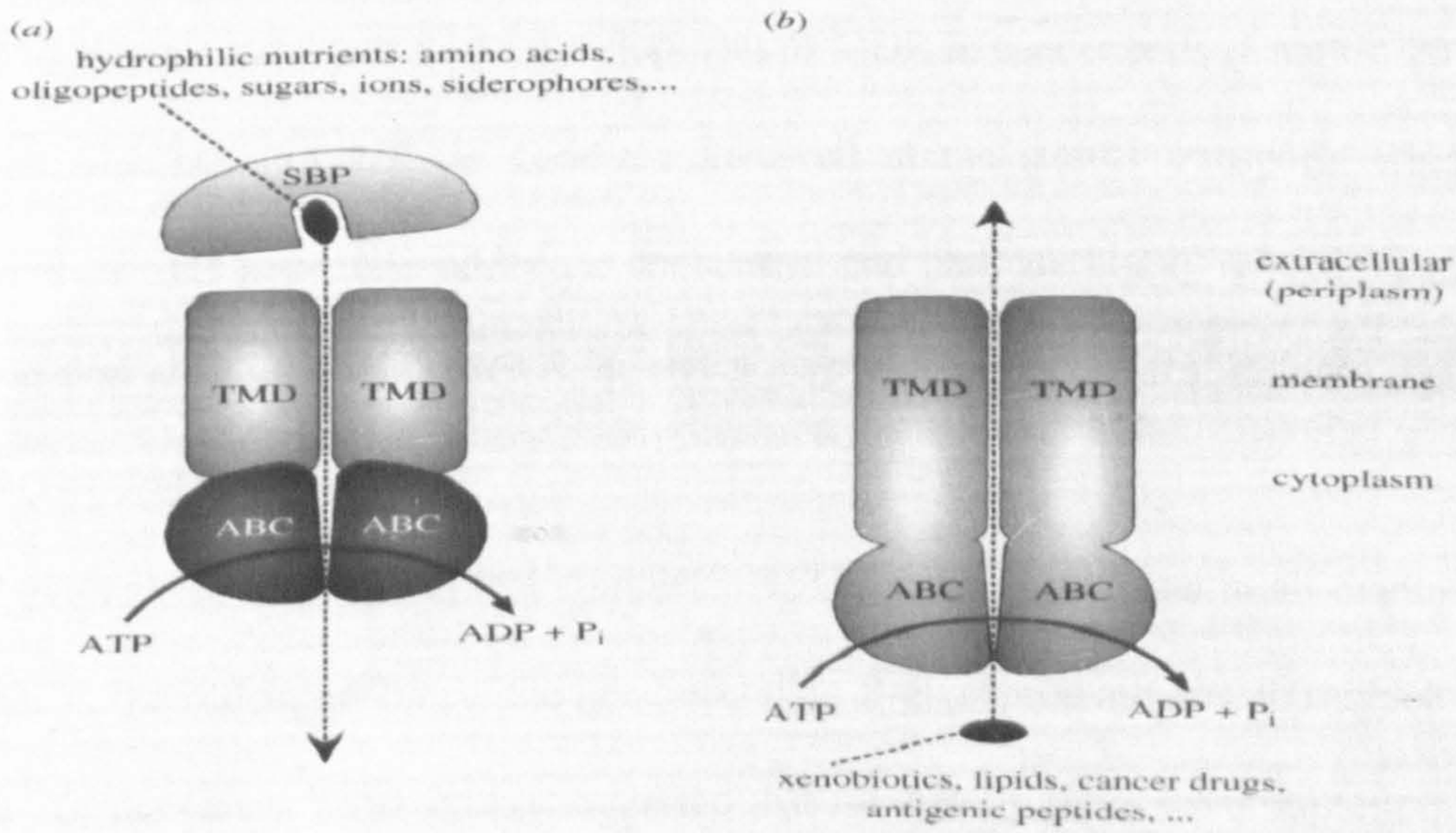

Figure 2.1: General structure of ABC proteins (Retrieved from: http://d1vn86fw4xmcz1.cloudfront.net/content/royptb/364/1514/239/F1.large.jpg)

2.2 ABC Superfamily

ATP-binding cassette protein superfamily consist a large number of transporters, channels and regulators in both prokaryotes and eukaryotes. Based on the study of 3D structure on the ABC transporter in bacterial, the two NBFs (nucleotide binding folds) come together in a head-to-tail fashion to form an ATP-binding domain using residues from both domains. The proteins in the membrane were anchored by the hydrophobic domains and provide the channel to transport the substrate. This ABC gene superfamily is divided into eight different subfamilies based on the TMD and NBD sequence similarity. They are subfamily A, B, C, D, E, F, G, and H. Seven of them (subfamily A-G) are presence in human genome. There is 49 out of 58 members were found in human genome and the rest are found in animal species by which 68% of them are expressed in vertebrates' genome (Dean & Annilo, 2005).

At first, ABC transporters were categorized to exhibit nutrient uptake system. But as we acknowledged now, it has been proven that many ABC transporters are exporters. In fungi, the ABC transporters grant the resistance to antifungal agents by withdrawal of such agents out of the cells. (Higgins, 2001) Apparently, *ABCB1, ABCC1*, and *ABCG2* gene has appeared to play a crucial part in the development of MDR in cancer cells (Charles, 2013). Meanwhile, *ABCG5* and *ABCG8* gene are involved in the sterols transports whereas *ABCG1* and *ABCG4* gene induced by the cholesterol and both are highly related transport proteins. It was suggested that *ABCC3*, 4, and 5 involved in biochemical defence against toxicants (Luckenbach et al., 2014).

Previous study had found that completes *ABCB* genes is identical in all mammals, except that in the rodent genomes, the gene encoding P-glycoprotein (*ABCB1)* which has two copies of the genes. Thus, it was suggested that the ABCB genes have important and conserved functions that are similar in all mammals (Dean & Annilo, 2005). To date, there

are ten of the human ABCB genes have orthologs in zebrafish genome and the only genes that are absent are *ABCB4* and *ABCB5* genes. However, even though the ABCB1/MDR1 or ABCC7/CFTR have been studied widely and their functions has been notated, the roles of many others ABC gene are still unknown (Anillo et al., 2006).

2.3 ATP-Binding Cassette Subfamily H member 1 (*ABCH1*)

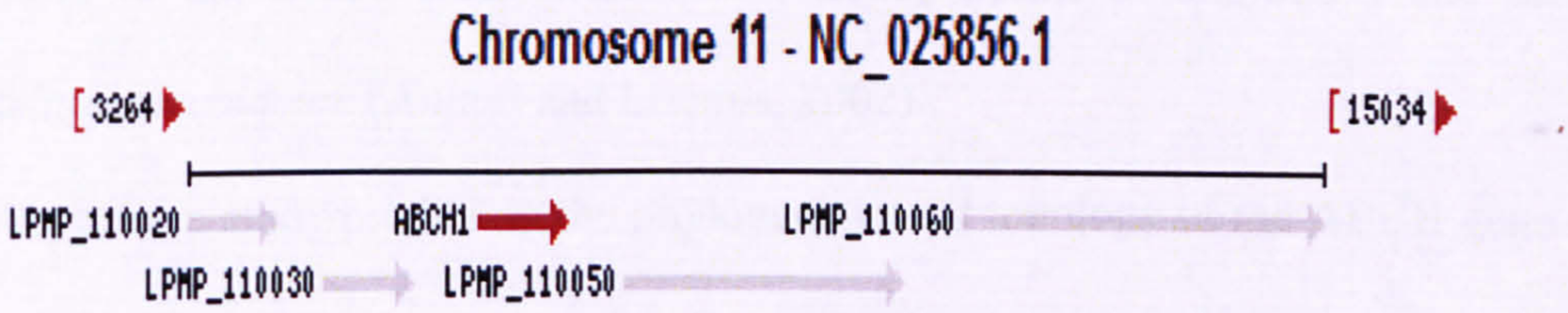

Figure 2.2: The chromosome 11 which *ABCH1* gene falls to. The red arrow shows the location of the *ABCH1* gene in *Leshmania panamenis*, a blood parasite that cause leshmaniasis disease in humans and rodents (Retrieved from: http://www.ncbi.nlm.nih.gov/gene/22572869)

The figure above shows the chromosome 11 (NC_025856.1) in *Leshmania panamenis*, a blood parasite that cause leshmaniasis disease in humans and rodents. The *ABCH1* gene falls between the 6294 to 7199 base pair with transcribed region size of 905 base pairs. The *ABCH1* gene is also found in chromosome 6 of *Dictyostelium discoideum AX4* (National Centre for Biotechnology Information).

Unlike those seven subfamily, subfamily H can generally be found in vertebrates such as teleost and mammals but not in human. *ABCH1* gene is common among vertebrates but unique to fish. Dean and Anillo (2005) reported that ABCH transporters are equivalent to members of the ABCG subfamily which is a ‘‘half transporters”.

The first identified *ABCH* gene was not in mammals but in the genome of fruit fly, *Drosophila melanogaster*. Genome sequencing of *D. melanogaster* and human genome were done to examine the ABC proteins range and a new branch on the calculated phylogenetic tree which called as *ABCH* was discovered (Dean *et al.*, 2001; Roth et al.,

2003). However, the previous study show that there is no known physiological function associated with any of this gene found in insects (Dean and Anillo, 2005). Another study by Popovic et al. (2001), reported that the function of *ABCH1* gene is not yet well known in fish. So far, ABCH member have been reported presence on the genome level in teleosts (zebrafish and green spotted pufferfish, *Tetraodon nigroviridis*), insects (fruitfly *Drosophila melanogaster*, *Daphnia pulex*, *Anopheles gambiae* and silk moth *Bombyx mori*) (Roth et al., 2003; Dean and Anillo, 2005; Sturm et al., 2009) and amoeba *Dictyostelium discoideum* (Anjard and Loomis, 2002).

Anyhow, a study predicting the phylogenetic and topology of the ABCH gene done confirmed that this gene is closely related to ABCG gene although they shares only 12-14% amino acid sequence identity (Popovic et al., 2010). Furthermore, the predicted topology of *ABCH1* showed that they are similar to ABCG subfamily but differs in the loop arrangement. This indicates that whether the *ABCH1* is a part of sterol transport like *ABCG1* or multixenobiotic defence like *ABCG2*.

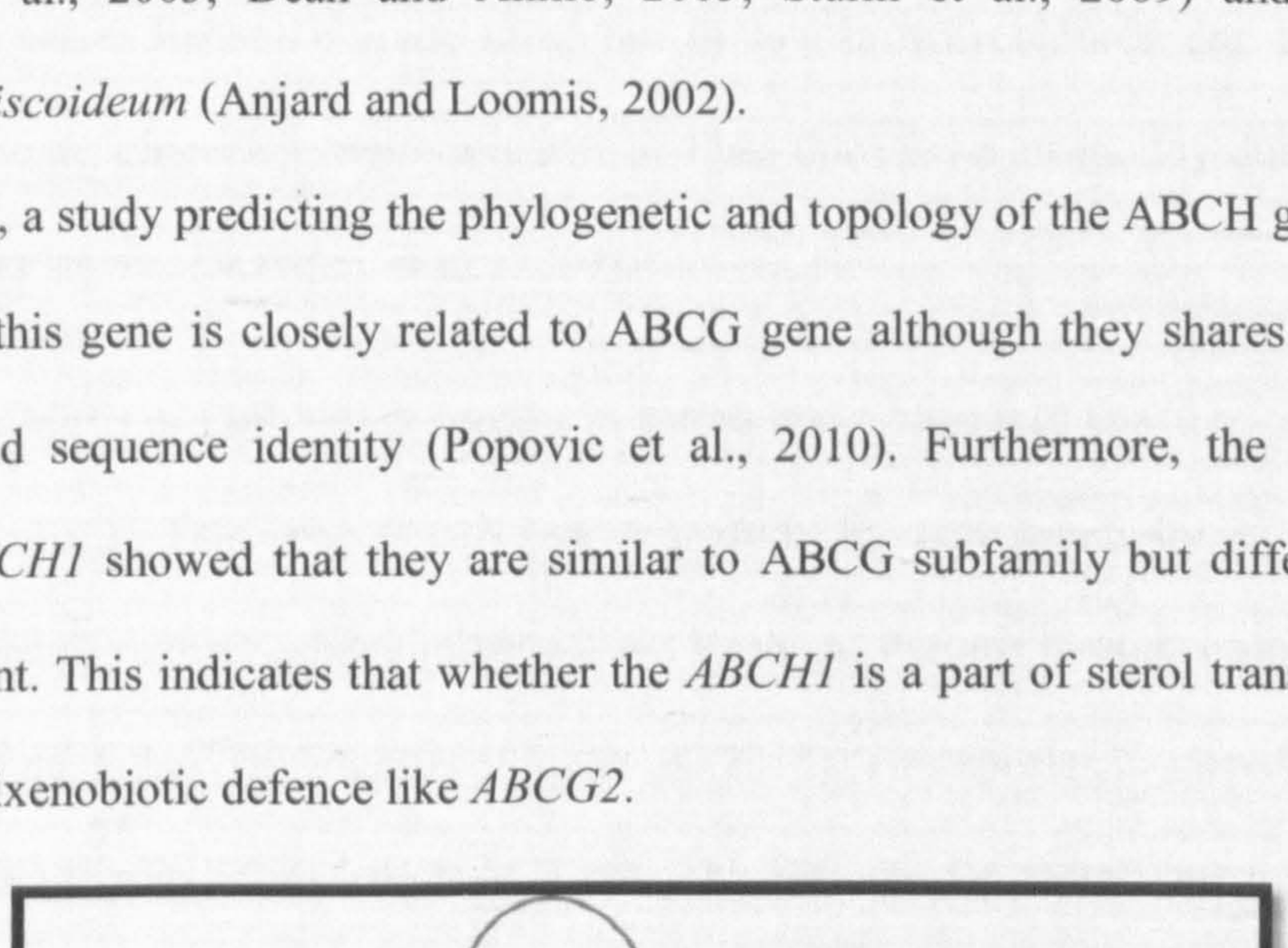

Figure 2.3: The predicted topology of *ABCH1* (Adapted from Popovic *et al.*, 2010)

2.4 *Rasbora Sarawakensis*

Rasbora fish is a tropical freshwater in family of *Cyprinidae* and is natively found in the island of Borneo. Rasbora fish can also be found in West Kalimantan, Indonesia. Its presence had been verified from several river systems including the Batang Kayan and Sungai Sarawak in Sarawak and the Mempawah and Melawi in West Kalimantan. (Liao et al., 2010) The native habitat of Rasbora fish is non-fast forest streams with thick edge of vegetation. The water habitat is quite clear but in low brown coloured due to tannins released from the decomposing organic matter and has thick layer of muddy due to fallen tree branches and leaves. (Mayden, et al., 2007).

R. sarawakensis is a schooling species in nature which they will stay in a group of at least 8-10 individuals. This species can live peacefully in the community of Southeast Asian-Indian species such as other similarly-sized Rasbora, Puntius, botiid/cobitid loaches and gouramis of the genus Trichogaster (Liao et al., 2010). This species is a small size fish which can reach the maximum length of 5 cm. The features for males and females are different which matured females can be noticed by their rounder-bellied and might be a bit larger than males.

Suitable temperature, pH value, and hardness are the aspects that should be considered for the water conditions as well as the diet of the fish when keeping fish in aquarium. For this species, the optimum temperature is 22 – 26°C. The pH value should be in the range of 6.0 to 7.5 and the hardness should be in a range of 2 to 12°H. In the aquarium, *R. sarawakensis* should be fed with dried foods of suitable size. Best coloration and encouragement for the fish to come into breeding condition can be achieved by giving it daily meals of small live and frozen foods such as *Daphnia*, and bloodworm.

R. sarawakensis share some characteristics with the same family of the different genus namely, *Danio Rerio* (zebrafish). Both species are small in size, short lifecycles, has rapid growth, produce large number of progeny, and zebrafish embryos are transparent which allows scientist to study their developmental stages. *Rasbora Sarawakenis* will be used as a model in this study because their gene is less discovered, has unique characteristics like zebrafish, and they has the potential to become the ecotoxicological model as it is endemic to Borneo Island.

Figure 2.4: *Rasbora sarawakensis* (Adapted from *Rasbora sarawakensis*, 2012)

Figure 2.5: The habitat of *R. sarawakensis* near the Batang Sadong, Sarawak, Borneo (Adapted from *Rasbora sarawakensis*, 2012)

3.0 MATERIALS AND METHODS

3.1 List of Materials

TRI reagent (Sigma, USA)

Chloroform

Isopropanol

TAE (Tris-acetate EDTA) buffer

EasyScript® Reverse Transcriptase (TransGen, China)

Wizard SV Gel and PCR Clean-Up System Kit (Promega,USA)

0.1 M $CaCl_2$

Glycerol solution

pGEM®-T Easy Vector (Promega, USA)

LAIX (LB agar/ Ampicillin/IPTG/X-Gal

Pure Yield Plasmid Miniprep System Kit (Promega, USA)

3.2 Maintenance of *Rasbora sarawakensis*

Fish will still show good coloration even growing up in a well-planted arrangement with a dark substrate, thus the decoration for the habitat of *Rasbora sarawakensis* was not really fastidious. For the tank décor, some floating plants were put to reduce the amount of light entering the tank. Then, filtration were set up to reduce the frequent changing the water tank, which done only once a week. The temperature of the water was in the range of 22-

26°C, while the pH is within 6.0 to 7.5 and hardness from 2 to 12°H. The *Rasbora sarawakensis* are fed twice a day with suitable food.

3.3 Primer Design

Since only one species being used to design the primer for *ABCH1* gene, coding sequence for *ABCH1* gene in zebrafish was used as a template in ExPASy Scan Prosite (www.expasy.scanprosite.com) to design primer which was based on the TMD and NBD regions of ABCH transporter. Next, Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was used to find out whether the sequence selected is suitable or not. The Oligo calculate software (http://www.basic.northwestern.edu/biotools/oligocalc.html) was the software used to analyze all the suitable primer pairs designed for palindromes, hairpin, dimmers and temperature (Tm).

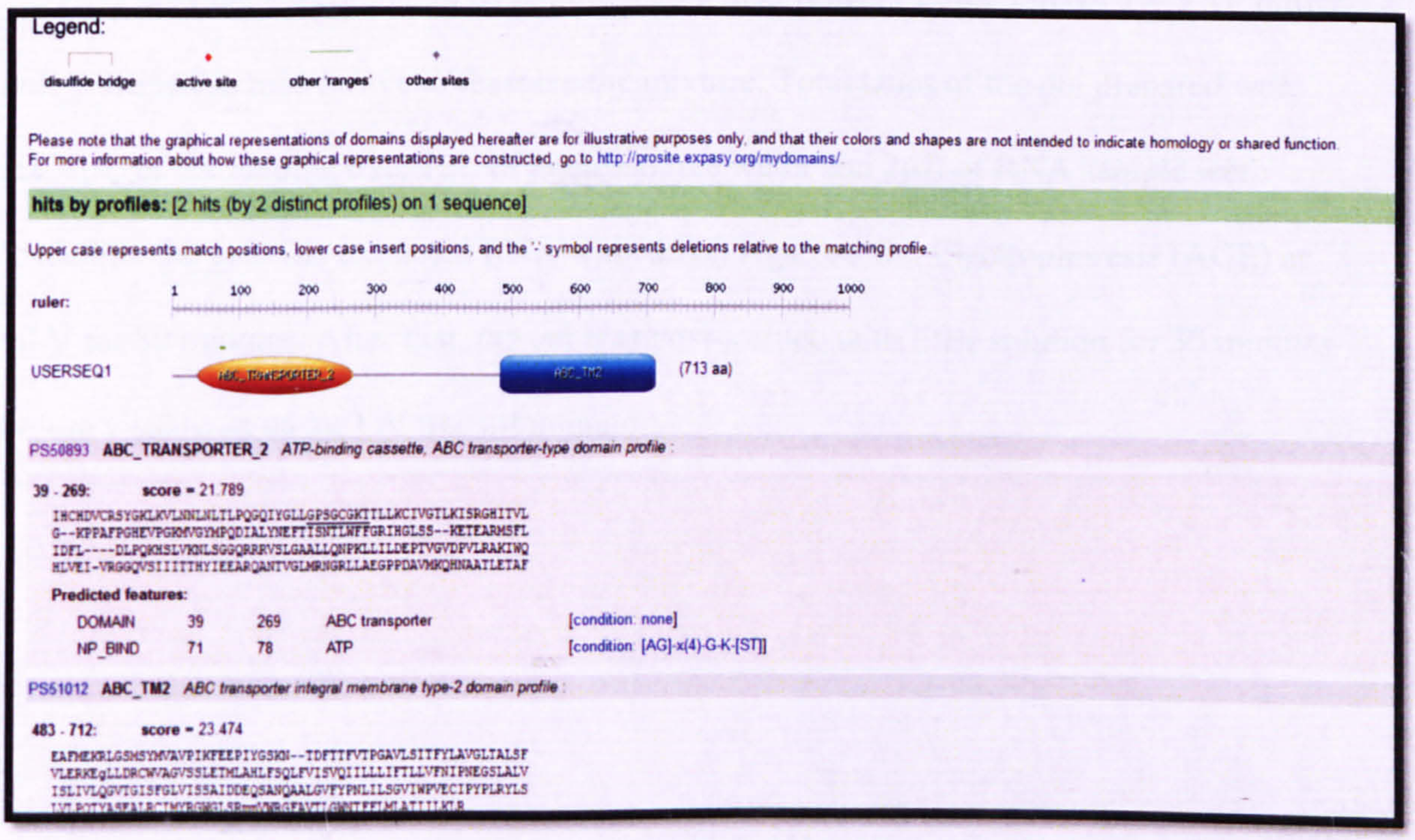

Figure 3.1: The ExPASy Scan Prosite shows the 2D structure of the TMD and NBD of the ABCH transporter

3.4 RNA Extraction

First, minced tissue of *Rasbora sarawakensis* were inserted into a 1.5ml micro-centrifuge tube and homogenized using the TRI reagent under the fume hood. The mincing of the tissue sample was done on ice to prevent enzymatic reaction which degrades the RNA. Homogenized tissue was centrifuge at 13 000 rpm for 10 minutes at 4 °C. Then, the supernatant were transferred into a new tube at leaved for 5 minutes at room temperature. Next, 200 µL of chloroform were added and the tube covered tightly before vigorously being shake for 15 seconds. The tube was leaved for 5-10 minutes at room temperature. The tube was centrifuged again at 13 000 rpm for 15 minutes at 4 °C. A white pellet (RNA) was formed at the bottom of the tube. Then, the supernatant was removed and the RNA pellet was wash with 1 mL of 75% ethanol. Tube was centrifuged at 13 000 rpm for 5-10 minutes. The ethanol was discarded and the pellet was air dried for 5-10 minutes. Lastly, the pellet was suspended with autoclaved water.

1% of gel was prepared by adding 0.25 g agar powder into 25mL of 1X TAE buffer before heated in microwave to dissolve the mixture. Total lanes of the gel prepared were six. 1µL of 5X loading dye, 2µL of nuclease free water and 2µL of RNA sample were loaded on the gel. The extracted RNA was run on Agarose Gel Electrophoresis (AGE) at 90 V for 30 minutes. After that, the gel was post-stained with EtBr solution for 30 minutes before visualized under UV transilluminator.